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Short Communication

Studies on Dilutors vis-a-vis Freezability of Patanwadi Ram Semen: II. Effect on Spermatozoal Enzyme Leakage *

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ABSTRACT

The effects of dilutors, rams, stages of freezing process and their interactions were studied in 30 ejaculates (6/ram) in a 3x5x3 factorial experiment for motility and leakage of spermatozoal enzymes during cryopreservation of Patanwadi ram semen. The 3 diluents used with 20% egg yolk and 4% glycerol were tris fructose citric acid egg yolk glycerol (TFCEG), phosphate buffer glucose fructose sodium citrate potassium chloride egg yolk glycerol –a synthetic phosphate medium (Phos) and sodium citrate glucose egg yolk glycerol (SCGEG). The effects of dilutors, rams, stages of freezing (initial, prefreeze and post-thawed) and their two-way interactions were highly significant on sperm motility and leakage of LDH, GOT and AKP enzymes. Minimum LDH leakage (383.2 ± 7.28 IU/billion sperms) and maximum post-thaw motility (36.67 ± 1.13%) was observed in TFCEG diluent followed by Phos (31.00 ± 1.43%) and SCGEG (19.83 ± 0.85%) diluents, although GOT and AKP leakage was minimum in Phos and maximum in TFCEG diluent. Dilutor x ram x stage interaction was also significant for all 3 enzymes. Rams having better sperm motility at dilution stage could express equally good post-thaw motility in Phos diluent. Further, the observations indicated that though motility was significantly higher in TFCEG diluent, the remaining attributes, except LDH leakage, were more congenial for freezing process in Phos diluent.

Key words: Dilutors, Freezability, Dehydrogenase, Phosphatase, Transaminase, Ram semen.

INTRODUCTION

Diluent plays an important role in optimum post-thaw recovery of spermatozoa (Mathur *et al.* 1991). Several reports pertaining to different media used for extending ram spermatozoa for successful pellet and ampoule freezing have appeared (Salamon and Lightfoot, 1969; Mathur *et al.* 1991; Ingole *et al.* 1999), but those of the comparative efficacy of preservability in straw are scare, particularly with reference to enzyme leakage and fertility (Sivaselvam *et al.* 2000; Mathur, 2007). Vulnerability of ram spermatozoa to dilution, cold shock, change in pH and composition of media and intolerance to glycerol pose practical problems in preservability in straw leading to lower post-thaw recovery rate (Colas and Brice, 1975; Mathur, 2007). Enzyme leakage has generally been recognized as a cellular injury, whereby membrane becomes inactivated or damaged resulting in the loss of cellular material (Kaya *et al.* 2001). Monitoring effect of cold shock and deep freezing in most of the domestic animals is possible by studying intracellular enzyme release (Pace and Graham, 1970; Ingole *et al.* 2000). Hence, this study was attempted to evaluate the interaction of semen of individual rams with different diluents for preservability in straws in terms of motility and enzyme leakage.

This study was conducted on 30 semen ejaculates of 5 mature Patanwadi rams maintained at AICRP on sheep breeding, GAU, SKNagar, North Gujarat. The rams were maintained under uniform nutritional and managerial regime in semi-intensive system. Semen was collected at weekly interval from each ram in separate AVs. Immediately after collection, the samples were kept at 30°C in water-bath, evaluated for

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individual sperm motility and split-diluted with 3 extender formulations each with 20% egg yolk and 4% glycerol, viz. (i) Sodium citrate glucose egg yolk glycerol –SCGEG (Salamon and Lightfoot, 1969), (ii) Phosphate buffer with glucose fructose sodium citrate potassium chloride egg yolk glycerol (Phosphate-synthetic medium) – Phos (Watson and Martin, 1975), and (iii) Tris fructose citric acid egg yolk glycerol –TFCEG (Kandasamy *et al.*, 1989). Before glycerolization, each dilutor was centrifuged at 8000 rpm for 10 min at room temperature and the supernatant was used for dilution of semen.

Split-ejaculates of individual rams after first step dilution (1:3) at room temperature (30°C) were cooled to 5°C over 1.5 hr and then equilibrated for 5 hrs at the same temperature in the refrigerator. In the second step of dilution, equal volume of first step diluted pre-cooled semen and glycerolated buffer (at 5°C) was mixed so as to maintain 150 million sperm per straw. French medium straws (0.5 ml) of different colour markings were then filled and sealed with PVA powder. The straws were frozen in liquid nitrogen vapour in a thermocoal box using standard freezing protocol, as recommended by Sahni and Mohan (1988). The straws were thawed after 24 hrs of freezing at 37°C for 15 seconds in water-bath. Each sample was studied for sperm motility, as well as leakage of lactate dehydrogenase, gluatmic oxaloacetate transaminase and alkaline phosphatase in freshly diluted, equilibrated and post-thawed semen. The samples were centrifuged in a refrigerated centrifuge at 8000 rpm for 20 min and the supernatant decanted was stored at –20°C till analyzed within a week. The estimations were performed using Span Diagnostic Kits as per the methods recommended therein. The data were analyzed statistically using a 3x5x3 factors factorial RBD (Snedecor and Cochran, 1986).

The findings of sperm motility and the activities of enzymes LDH, GOT and AKP in the seminal plasma of individual ram semen at 3 stages of freezing process in 3 diluents are presented in Table 1.

Sperm motility

Sperm motility was observed to be the highest overall and for individual ram semen in TFCEG diluent followed by Phos and SCGEG diluents at all three stages of freezing process (initial, after equilibration, post-thawed). The effects of all three variables, viz., dilutors, rams and stages of freezing over sperm motility were highly significant ($P<0.01$). The significant influence of ram on motility in 3 diluents studied suggested a definite interaction of ram with dilutor (Table 1). Post-thaw behaviour of spermatozoa of rams with varying motility in different dilutors suggested that ram semen have better survivability in decreasing order in TFCEG, Phos and SCGEG. Further, data show that though the initial sperm motility was highest in phosphate dilutor, the effect of equilibration/glycerolisation and freezing-thawing was much detrimental in SCGEG and Phos dilutors than the TFCEG. Findings suggest that TFCEG is the best diluent for straw freezing of ram semen as far as post-thaw motility is concerned.

The post-thaw motility obtained with TFCEG diluent in this study (36.67 ± 1.37%) is comparable to that reported by Lopez *et al.* (1988) and Kandasamy *et al.* (1989). Significant reduction in the motility of sperms in TFCEG diluent with different stages of freezing process is comparable with the reports of Haranath *et al.* (1982), Kalatharan *et al.* (1986) and Ingole *et al.* (1999). The overall post-thaw motility in Phos dilutor (31.00 ± 1.44%) is comparable with that of Varnavaskii *et al.* (1989). Significant effect of ram over the sperm motility found in the present study corroborated with the report of Kalatharan *et al.* (1986). Significant variation observed in post-thaw motility among different diluents also coincided with the reports of Kalatharan *et al.* (1986) and Awad (1999). Mathur *et al.* (1991) and Aisen *et al.* (2002) concluded that membrane protecting disaccharides confer grater cryoprotective capacity to the base extenders.

Leakage of lactate dehydrogenase

The effect of dilutors, rams and stages of freezing process for LDH leakage was highly significant ($P<0.01$). Maximum leakage was observed in Phos diluent followed by SCGEG and the least in TFCEG. The interactions of dilutors with rams and stages of freezing process ($P<0.01$) and rams with stages ($P<0.05$) were significant. The leakage of LDH was significantly and positively correlated with the leakage of AKP

Table 1. Sperm motility (%) as well as leakage of spermatozoal enzymes at different stages of freezing process of Patanwadi ram semen in three different extenders (Mean ± SE)

Trait	Ram No.	Stages of freezing process and extenders								
		On dilution			After equilibration			Post-thawed		
		TFCEG	Phos	SCGEG	TFCEG	Phos	SCGEG	TFCEG	Phos	SCGEG
Sperm motility (%)	P1	75.00±2.45	75.83±4.54	68.33±2.79	65.00±2.24	62.50±2.81	47.50±2.50	39.17±2.71	35.83±1.54	19.17±1.54
	P2	80.00±1.29	76.67±1.67	75.00±1.83	65.83±2.01	62.50±3.35	51.67±4.77	40.83±2.39	34.17±2.39	21.67±2.71
	P3	68.23±2.79	70.00±2.89	64.17±2.01	55.00±4.28	55.00±3.65	45.00±2.24	32.50±2.14	25.83±2.39	16.67±1.05
	P4	77.50±2.14	79.17±2.01	70.00±2.24	68.33±2.11	64.17±3.75	49.17±2.71	39.17±1.54	37.50±2.50	22.50±2.14
	P5	61.67±2.79	64.17±2.01	62.50±3.09	50.00±2.58	45.00±2.58	44.17±2.01	31.67±1.67	21.67±1.05	19.17±2.01
	Av.	72.50^{a}±1.57	73.67^{a}±1.32	68.00^{b}±1.30	60.83^{a}±1.75	57.83^{b}±1.90	47.50^{c}±1.35	36.67^{a}±1.13	31.00^{b}±1.43	19.83^{c}±0.85
LDH (IU/billion sperm)	P1	38.93±3.48	69.24±4.49	44.37±5.56	106.97±4.06	190.30±11.38	115.17±11.44	386.76±19.34	597.95±14.48	404.75±31.26
	P2	49.02±0.97	74.20±4.77	50.43±4.61	99.89±4.26	173.24±7.74	122.79±12.27	376.67±17.42	497.89±10.69	392.74±19.13
	P3	40.50±3.11	66.77±3.32	49.27±3.13	104.78±45.09	148.91±8.86	112.49±10.87	401.04±20.37	482.33±29.78	433.29±29.94
	P4	46.10±3.45	58.45±2.02	46.19±1.40	100.71±6.89	127.53±6.45	105.58±3.73	356.74±12.17	432.48±20.63	411.20±20.71
	P5	44.16±2.70	65.48±3.26	48.14±3.23	95.48±5.84	135.64±8.76	100.01±5.79	389.71±5.72	418.53±26.48	426.87±16.91
	Av.	43.74^{b}±1.31	68.44^{a}±2.07	47.68^{b}±1.66	101.6^{b}±2.33	155.1^{a}±5.68	111.2^{b}±4.19	382.2^{c}±7.18	485.8^{a}±14.81	413.8^{b}±10.46
GOT (Units/ billion sperm)	P1	286.9±26.9	214.8±14.1	242.5±24.5	466.5±44.3	351.2±29.2	445.1±52.8	784.39±61.43	779.16±60.45	867.65±75.61
	P2	246.0±18.5	179.1±16.2	152.3±18.6	419.4±43.5	246.0±16.9	458.4±46.9	699.49±34.37	840.43±70.51	821.43±45.52
	P3	280.5±13.2	165.8±12.0	196.1±13.2	532.2±50.6	289.3±26.4	446.2±24.7	657.89±75.67	987.18±99.08	725.05±58.89
	P4	293.6±26.0	149.1±6.2	157.6±17.5	492.1±49.5	243.2±16.1	377.8±23.6	815.18±48.04	698.50±25.64	648.83±24.38
	P5	283.6±35.7	174.3±5.07	26.1±14.4	503.4±63.1	379.1±37.6	425.3±44.7	812.25±70.11	807.02±94.85	758.30±37.94
	Av.	278.1^{a}±1.8	170.5^{b}±8.1	194.9^{b}±10.1	482.7^{a}±22.2	301.8^{c}±15.0	430.4^{b}±15.5	813.8^{ab}±11.0	855.5^{a}±81.8	764.1^{b}±25.6
AKP (KAU/ billion sperm)	P1	815.1±93.8	674.9±48.1	611.2±63.6	1467.4±102.7	1139.4±74.3	920.0±83.7	2642.2±141.1	2169.9±127.4	1904.0±53.2
	P2	395.5±55.5	390.2±34.9	531.4±61.0	806.8±20.9	644.1±43.1	803.1±52.9	1909.3±190.7	1549.4±90.5	1935.8±176.1
	P3	695.4±59.3	516.4±42.2	612.3±37.5	1127.9±19.4	819.3±63.4	879.4±76.5	2035.9±171.3	1720.5±158.3	2162.9±211.2
	P4	669.3±65.6	381.9±45.9	480.8±26.9	1007.2±142.9	523.8±87.5	766.9±57.9	1875.3±262.3	1382.9±117.2	1807.6±99.4
	P5	669.9±54.5	630.9±28.1	558.5±56.9	983.1±58.5	908.1±37.7	833.1±93.4	1711.6±166.3	1972.6±67.8	2268.6±208.9
	Av.	638.0±38.9	518.9±27.9	558.8±23.2	1078.4^{a}±53.7	833.6^{b}±40.8	840.5^{b}±32.5	2034.9^{a}±99.1	1760.1^{b}±70.9	2021.9^{a}±75.1

Means carrying similar superscript in a row within the stage do not differ significantly between dilutors. Stage variation P<0.01 for all traits.

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during freeze-thaw process (r = 0.27). LDH is released in to the extra-cellular medium even with mild shock to spermatozoa leading to alteration in the per cent motility or morphology (Aguirre *et al.* 1988; Ingole *et al.* 1999) as reflected in this study. Ozinowo (1981) reported that the LDH enzyme is released from spermatozoa in the extra-cellular medium even in the absence of any apparent cellular damage. Findings of the present study also corroborated with the report of Ingole *et al.* (2000) that Tris diuent could provide maximum protection to the ram spermatozoa in terms of LDH leakage as compared to raffinose and lactose egg yolk glycerol diluents. They also observed significant variation between stages in the leakage of enzyme and further suggested that lower the amount of leakage, better is the semen or dilutor quality.

Leakage of glutamic oxaloacetic transaminase and alkaline phosphatase

The analysis of variance revealed significant (P<0.01) variation in the leakage of GOT and AKP between dilutors, rams and stages of freezing process. The leakage of both the enzymes in extra-cellular medium was maximum in TFCEG followed by SCGEG and minimum in Phos diluent. The interactions of dilutors with rams and stages of freezing process were also highly significant. However, rams and stages of freezing had no significant interaction. The leakage was accentuated by the stages of freezing process, since at each stage a different type of injury was inflicted to the spermatozoa leading to their damage and hence the three-way interaction of ram x dilutor x stage also revealed significant variation in the GOT –AKP leakage (Table 1).

Significant increase in extra-cellular activities of transaminases and alkaline phosphatase observed at post-thaw stage over initial or prefreeze values corroborated with the reports of Ingole *et al.* (1999, 2000), Sivaselvam *et al.* (2000) and Kaya *et al.* (2001) in ram and/or buck semen. The amount of GOT release is proportional to the damage in sperm cells (Pace and Graham, 1970). The damage to spermatozoa may occur due to diluent and cold shock, glycerol levels, type of diluents, osmolarity and stages of freezing process (Pareek *et al.* 1981; Mathur, 2007). Thus, the concept of effect of diluent and stages of freezing influencing the amount of GOT and AKP leakage in extra-cellular medium corroborated with the observations of Ingole *et al.* (1999) and Kaya *et al.* (2001).

Further, the significant influence of rams on the GOT leakage is supported by the findings of Pace and Graham (1970), Aguirre *et al.* (1988) and Sivaselvam *et al.* (2000). The semen quality of individual ram is an inherent capability expressed as its interaction with environment in terms of fertility and vulnerability to shock by individual ram sperms differ as evidenced by the release of GOT from spermatozoa in extra-cellular medium. Vorobev (1980) and Kaya *et al.* (2001) also reported significant difference in AKP activity of ram sperms at different stages of freezing process corroborating the findings of present study.

Thus, it could be concluded that though motility was significantly higher in TFCEG diluent, the remaining attributes, except LDH leakage, were more congenial for freezing process in Phos diluent.

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